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Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants

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ABSTRACT

A two-component cloning system to transfer foreign DNA into plants was derived from the octopine Ti plasmid pTiB6S3. pGV2260 is a non-oncogenic Ti plasmid from which the T-region is deleted and substituted by pBR322. pGV831 is a streptomycin-resistant pBR325 derivative that contains a kanamycin resistance marker gene for plant cells and a site for cloning foreign genes between the 25-bp border sequences of the octopine T-region. Conjugative transfer of pGV831 derivatives to Agrobacterium and cointegration by homologous recombination between the pBR322 sequences present on pGV831 and pGV2260, can be obtained in a single step. Strains carrying the resulting cointegrated plasmids transfer and integrate T-DNA into the genome of tobacco protoplasts, and transformed tobacco calli are readily selected as resistant to kanamycin. Intact plants containing the entire DNA region between the T-DNA borders have been regenerated from such clones. In view of these properties we present pGV831 and its derivatives as vectors for efficient integration of foreign genes into plants.

INTRODUCTION

Agrobacterium tumefaciens integrates T-DNA, a segment of its tumor-inducing plasmid (Ti plasmid), into nuclear plant DNA. T-DNA expression in plant cells leads to tumorous cell proliferation known as crown gall (for a review, see [1-2])

Agrobacterium can be used as a vector to integrate foreign DNA into dicotyledonous plant cells [3] and recent data suggested that this can be extended to at least some monocotyledonous plants [4-5].

Important progress has been made to facilitate the use of the Ti plasmid as a vector for plant genetic engineering. Mutational analysis of the Ti plasmid has shown that expression of T-DNA genes is not required for transfer and integration of T-DNA [6-8]. Small, directly repeated sequences which flank the T-DNA (border sequences) were suggested to play a key role in T-DNA integration. Indeed, introduction of a short synthetic DNA, identical to such a border sequence, is sufficient to restore T-DNA integration from a Ti plasmid that lacks one end of the T-DNA [9]. These

observations were the basis for the development of efficient non-oncogenic Ti plasmid vectors from which the tumor genes are removed by an internal deletion in the T-DNA. These Ti plasmids still contain the border sequences and consequently transfer T-DNA without tumor induction [10-11].

One such plasmid, pGV3850 [7] has proven very useful. It contains a substitution of the internal T-DNA genes by the commonly used cloning vehicle pBR322. Plant cells transformed by pGV3850 have the same regenerative capacities as untransformed cells and several procedures have been developed to obtain intact plants that contain the pGV3850 T-DNA [10, 12]. Transformed tissues are identified by screening for nopaline synthase activity, which is encoded by a gene still present in the pGV3850 T-DNA. pGV3850, through the pBR322 sequences present in its T-DNA, is an efficient acceptor plasmid for gene transfer experiments to plant cells. Indeed, genes cloned in pBR-like plasmids are transferred and inserted via a single homologous recombination event into the pGV3850 T-DNA.

Another major advance in the application of T-DNA as vector is the use of plant promoter sequences to express chimeric genes in plants [13]. Using the nopaline synthase promoter and bacterial antibiotic resistance genes, dominant selectable markers for plant cells have been constructed [14-16].

In this paper we describe an octopine Ti plasmid-derived vector system for gene transfer to plant cells. Its advantages over other vector systems, such as pGV3850, will be discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are listed in Table 1. Media and culture conditions were as previously described [23]. Conjugations involving *E. coli* and *Agrobacterium* were performed according to [25].

DNA manipulations

Total plant DNA was prepared as described previously [7]. All restriction endonuclease digests were incubated in TA buffer [26]. Other methods were as described [27].

Plant cell culture methods

The cocultivation method was first described by [28] and slightly modified according to [14]. Km^R calli were selected in liquid or in solid medium as described previously [10].

The phenotypic expression of the kanamycin resistance gene in plant

Table 1. Bacterial strains and plasmids

	Antibiotic resistance	Relevant Characteristics	Origin
Bacterial strains			
E. coli			
K514		*thr*, *leu*, *thi*, *hsdR*	[17]
SK383	Str	*arg*, *his4*, *ilv*, *lac*MS286, Str^R	S. Kurshner
A. tumefaciens			
C58C1Rif^R	Rif	Rif^R derivative of C58, cured for pTiC58	[18]
Plasmids			
pB322	Cb, Tc		[19]
pBR325	Cb, Cm, Tc		[20]
pHC79	Cb, Tc	cosmid	[21]
pKC7	Cb, Km	pBR322 derivative carrying 1.8 kb *Hin*dIII/*Bam*HI fragment of Tn*5*	[22]
pGV600	Cb, Tc	pBR322 derivative lacking *Bam*HI site	[23]
pGV99	Cb, Cm	pBR325 derivative carrying 4.71-kb *Bam*HI fragment 17 of pTiAch5	[24]
pGV700	Cb, Cm	pBR325 derivative containing the 1-kb *Hin*dIII/*Bgl*II part of the *Hin*dIII-18 fragment of pTiAch5 and the 6.5-kb *Bgl*II/*Hin*dIII part of *Hin*dIII fragment 1 of pTiAch5 (see Figure 1)	This work
R64*drd*11	Sm, Tc	transfer-derepressed derivative of Iα-type plasmid	[25]
pGJ28	Km	ColD replicon carrying ColE1 *mob* and *bom*	[25]
pGV2217	Km	substitution mutant of $pTiB6S3Tra^C$ lacking the entire TL-region	[6]
pLGV2381	Cb	pBR322 derivative carrying *nos* promoter	[14]

Abbreviations : Cb, carbenicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampicin; Str, Sm, streptomycin; Tc, tetracycline.

tissues was assayed (J.-P. Hernalsteens, personal communication) by placing leaf segments (1 cm^2) on Murashige and Skoog medium [29], supplemented with 0.8% agar, 0.5 mg/l 2,4 D, 0.5 mg/l BAP, and 50 µg/ml kanamycin. Callus induction from control plants is completely inhibited at this concentration of kanamycin, whereas leaf tissue from plants containing the chimeric Km^R gene from pGV831 readily developed callus after 2 to 3 weeks.

RESULTS

The acceptor Ti plasmid pGV2260

The Ti plasmid pTiB6S3 contains two adjacent T-regions, TL (T-left) and TR (T-right) [30]. In order to obtain a mutant Ti plasmid lacking the TL- and TR-region we constructed the intermediate vector pGV746. pGV746 (Figure 1) is a pBR322 derivative which contains the two Ti plasmid fragments that are located respectively to the left and outside of the TL-DNA segment, and to the right and outside of the TR-DNA sequence. Double recombination between pGV2217, a pTiB6S3 derivative containing a Km^R marker, and pGV746 resulted in pGV2260 (Figure 1). The entire TL- and TR-region is deleted in pGV2260 and substituted by sequences derived from pBR322. pGV2260 is used as acceptor Ti plasmid in further experiments.

Construction of pGV831

The different steps in the construction of pGV831 are outlined in Figure 2. The vector part contains a portion of pBR325 and an additional Sm resistance gene. This marker is important to allow efficient selection for cointegration with the acceptor plasmid pGV2260 in *Agrobacterium*.

The T-region of pGV831 consists of the DNA regions surrounding the left (1015 bp) and right (500 bp) border sequences of the TL-DNA. These sequences flank a *neo* gene under control of the *nos* promoter which provides a dominant kanamycin resistance in plant cells [14]. pGV831 has a unique *Bam*HI site for cloning into the T-region.

Cloning into pGV831

As a model experiment, the octopine synthase (*ocs*) gene was inserted into pGV831. The 1635-bp *Rsa*I fragment from pGV99 [24] was purified and inserted into the *Hinc*II site of the M13mp7 vector [32] to produce mGV1. The *ocs* gene from mGV1 was isolated as a *Bam*HI fragment and introduced into the *Bam*HI site of pGV831 to produce pGV833 (Figure 3). This cloning regenerates the original 5' end sequence of the *ocs* gene as found in pTiB6S3. The *neo* gene and the *ocs* gene are positioned in the same orientation.

pGV833 was introduced into the acceptor Ti plasmid pGV2260 by a single

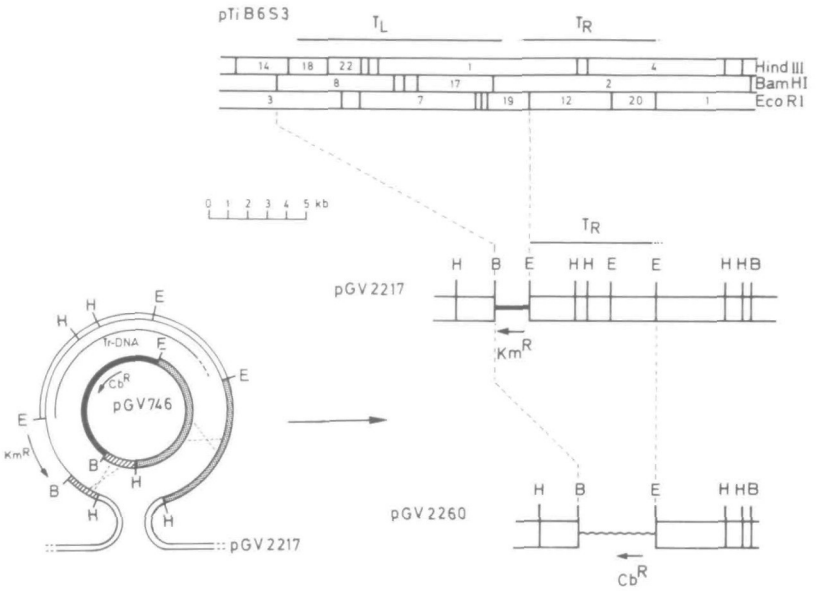

Figure 1. Construction of pGV2260.
Restriction map of the T-region of pTiB6S3 and pGV2217. In pGV2217 [6], the TL-region is substituted by a Km^R marker. The intermediate vector pGV746 was constructed as follows : the 2.3-kb HindIII/BamHI fragment from pTiAch5 fragment HindIII-14 ([///]) was cloned into pBR322, digested with HindIII and BamHI. This fragment is directly adjacent to the left of the TL-region. The resulting plasmid, pGV713, was selected as a $Cb^R Tc^S$ clone. The pTi-region adjacent to the right of the TR-region was cloned as a 4.2-kb EcoRI/HindIII fragment, derived from pTiAch5 fragment HindIII-4 ([·.·.] into pGV713 digested with EcoRI/HindIII. The resulting intermediate vector is pGV746. Recombinants between pGV746 and pGV2217 were isolated as Cb^R transconjugants after mobilizing pGV746 into C58C1Rif^R(pGV2217) using the technique described [25]. The double cross-over events between pGV746 and pGV2217, indicated by crossed lines, were obtained by screening the Cb^R transconjugants for the loss of the Km^R marker present on pGV2217. The physical structure of one Rif^R, Cb^R and Km^S transconjugant, pGV2260, was verified by Southern hybridization and is depicted in the figure.

homologous recombination, using the Sm^R gene of pGV833 as a selectable marker for cointegration. The mobilization of pGV833 from E. coli into Agrobacterium C58C1Rif^R(pGV2260) was performed according to [25]. Sm^R transconjugants appeared at 10^{-5} per recipient on MinA sucrose plates containing streptomycin (1000 µg/ml) and spectinomycin (300 µg/ml). The resulting Agrobacterium strains contain a pGV2260::pGV833 cointegrate plasmid. The structure of the T-region of one such strain, C58C1Rif^R (pGV2435), was confirmed by Southern blot hybridization (Figure 3). The

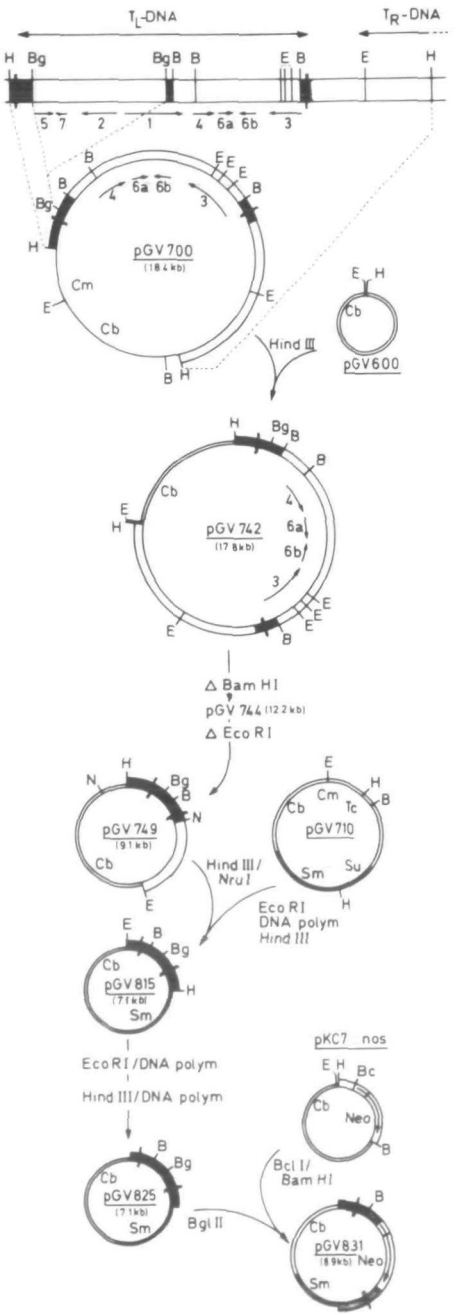

Figure 2. Construction of pGV831. The T-region of pTiB6S3 is presented on top of the figure. The dark fragments are those which are maintained in pGV831. The 7.5-kb HindIII fragment from pGV700 (Table 1) was recloned into pGV600, a pBR322 derivative lacking the BamHI site. The remaining TL-DNA genes in pGV742 were removed by deleting the internal BamHI fragments (pGV744). The left part of the TR-DNA was removed by deleting the internal EcoRI fragments (pGV749). pGV710 is a pBR325 derivative that contains an additional $Sm^R Su^R$ marker. To obtain pGV710 the 2.43-kb HindIII/PstI fragment from pBR325, containing the Cm^R gene, was cloned in a HindIII/PstI-digested cosmid pHC79 and the 1.62-kb BglII "cos" fragment of the resulting plasmid was substituted by a 3.45-kb BamHI fragment from the P-type plasmid R702 that encodes resistance to Sm/Sp and Su [31]. In order to obtain a fragment containing only the TL-border sequences, the 1.87-kb HindIII/ NruI fragment from pGV749 was cloned into pGV710 digested with EcoRI, and HindIII. The sticky ends obtained after EcoRI digest were flush-ended by treatment with Klenow DNA polymerase before HindIII digestion. pGV815 was isolated as a Sm^R, Cb^R, Cm^S and Tc^S clone. In pGV825 the EcoRI and HindIII sites were eliminated by filling-in the sticky ends and self ligation of the vector. A 298-bp BclI/ BamHI fragment from pLGV2381 [14] comprising the nopaline synthase promoter and cloned into the BclI site of pKC7 produced pKC7::nos. The nos promoter directs transcription of the neo gene in plant cells [14]. This chimeric Km^R gene was isolated as a BclI/BamHI fragment and cloned into the BglII site of pGV825 to produce pGV831. Abbreviations : B, BamHI; Bc, BclI, Bg, BglII, E, EcoRI; H, HindIII; N, NruI; Cb, carbenicillin; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; Su, sulfathiazol; Tc, tetracycline.

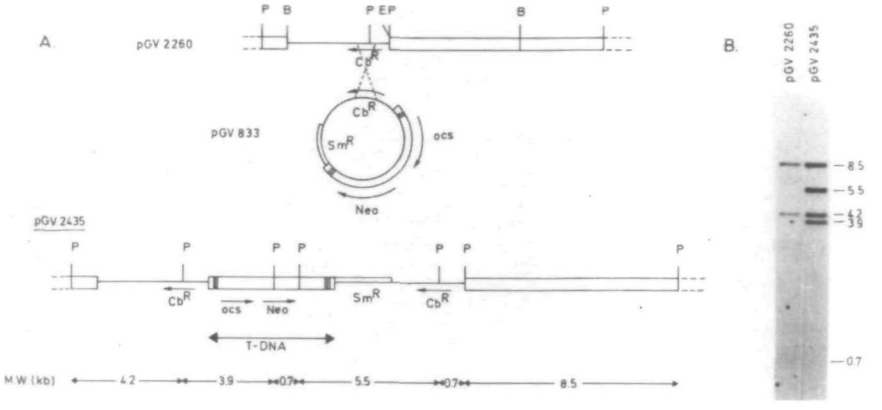

Figure 3. Southern blot analysis of receptor Ti plasmid pGV2260 and the pGV2260::pGV833 cointegrate pGV2435.
(A) Restriction maps of the region of pGV2260 which contains pBR322, pGV833 and the T-region of pGV2435.
(B) Southern blot analysis of total DNA of Agrobacterium strains C58C1Rif^R (pGV2260) and C58C1Rif^R(pGV2435) digested with PstI and hybridized against a pHC79 cosmid containing the entire pTiB6S3 TL and TR region, including BamHI fragments 8, 30, 28, 17 and 2 (see Figure 1).

T-region in pGV2435 is flanked by directly repeated pBR322 sequences (Figure 4A). pGV2435 was used in further experiments.

Protoplast transformation with C58C1Rif^R(pGV2435)

We transformed N. tabacum SR1 mesophyll protoplasts with C58C1Rif^R (pGV2435), selecting transformed calli for kanamycin resistance according to a procedure described by [10]. Resistant calli were selected either in liquid medium containing 25 or 50 µg/ml kanamycin sulfate, 20 days after protoplast isolation, or on solid medium with 50 µg/ml kanamycin, 40 days after isolation. Two independent infections were performed. Kanamycin-resistant calli appeared at frequencies of 1% and 2.9%, respectively on liquid medium with 25 µg/ml kanamycin; the frequency of colonies resistant to 50 µg/ml was 0.3% and 2.0%; selection on solid medium gave slightly higher results : 2.1% and 2.8%.

Transformed calli were further grown on solid medium containing 50 µg/ml kanamycin for 3 weeks and subsequently screened for octopine synthase activity as described by [23]. Twenty percent of the calli show ocs activity at a level comparable to that found in wild-type tumors, whereas variable but lower levels were found in 45% of the Km^R calli. No ocs activity could be detected in 35% of the calli.

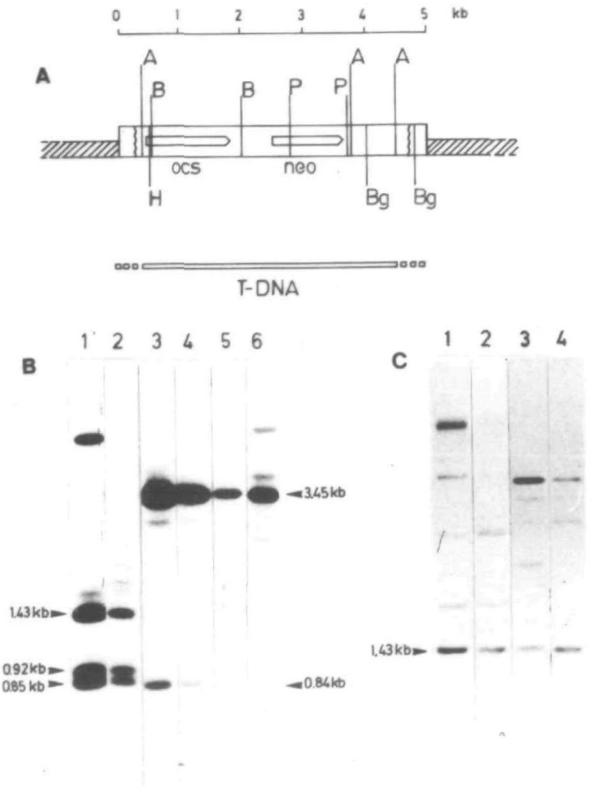

Figure 4. Southern blot analysis of pGV2435-transformed cell lines and regenerated plants.

(A) Restriction map of the T-region of pGV2435 : the hatched areas represent vector sequences, flanking the T-region in pGV2435. The waved lines indicate the TL-DNA border sequences. The bar represents the extent of the T-DNA in the different transformed lines, which have been analyzed.

(B) Hybridization pattern of DNA prepared from a Km^R and Ocs^+ cell line. Each lane contains 15 µg of plant DNA. DNA was digested with AccI (lane 6) and BamHI/PstI (lane 2). The HpaI/BglI fragment wich is internal in the pGV833 T-region was used as probe. Lane 1 is a 10-copy/tobacco genome reconstruction of BamHI-PstI-digested pGV833 (150 pg); lanes 3, 4 and 5 are respectively 10-, 5- and 2-copy/genome reconstructions of AccI-digested pGV833. The size of the T-DNA internal fragments is indicated.

(C) Lanes 1 and 2 show the hybridization pattern of BamHI-digested DNA prepared from two Ocs lines. The 1.43-kb fragment covers the ocs gene. Hybridization pattern of BamHI-digested DNA from a pGV2435-transformed callus line (lane 3) is identical with the pattern obtained with DNA from a plant regenerated from this callus (lane 4).

Abbreviations : A, AccI; B, BamHI; Bg, BglI; H, HpaI; P, Pst.

Regeneration of transformed plants

Calli from 5 different Ocs^+ and Km^R lines were transferred to solid Murashige and Skoog medium [29] containing 1 mg/l of 6-benzylamino purine to promote regeneration. Morphologically normal shoots were isolated and formed roots upon transfer to hormone-free medium. All regenerated plants produced octopine. Their resistance to kanamycin was confirmed by induction of callus tissue from leaf segments placed onto callus-inducing medium containing 50 µg/ml kanamycin (see Materials and Methods).

Physical analysis of the T-DNA structures

We determined whether the T-DNA border sequences, present in the pGV831-derived vectors, were used to delineate the ends of the T-DNA. As an example, figure 4B shows the hybridization pattern of DNA prepared from an Ocs^+ pGV2435-transformed cell line, probed with the α^{32}P-labeled HpaI/BglI fragment which covers most of the T-region of pGV2435. Hybridizing bands in a BamHI-PstI digest (Figure 3B, lane 2) have the size of the expected internal T-DNA fragments of 1.43, 0.92, and 0.85 kb. An AccI digest (Figure 3B, lane 6) reveals a 3.45-kb hybridizing band which contains the ocs and neo genes, as well as a 0.84-kb fragment (only detected after prolonged exposure). Using pBR322 as a probe, no hybridization could be detected (data not shown). This fact, and the occurrence of the internal AccI fragments of the T-DNA, precisely localize the termini of the T-DNA in the 483-bp and 560-bp fragments which contain the known right and left border sequences, respectively. By comparing the hybridization bands with the bands obtained in the 2- and 5-copy reconstructions (Figure 4B, lanes 4 and 5) we estimate the number of integrated T-DNA copies to be 3 to 4. The T-DNA of two Ocs^- lines was also analyzed. The presence of the ocs gene was demonstrated by the hybridization of the 1.43-kb BamHI fragment that covers the entire ocs gene (Figure 4C, lanes 1 and 2). Thus, the lack of octopine synthase activity in this line was not due to the absence of the gene. Finally, the T-DNA organization in a regenerated plant was shown to be identical to that in the original callus cell line (Figure 4C, lanes 3 and 4).

It is worthwhile mentioning that the neo gene from pGV831 was expressed although a characterized polyadenylation site had not been added to the 3' end of the gene. Presumably, the relatively rich AT region at the 3' end of the gene provides the necessary sequences for termination and polyadenylation.

DISCUSSION

This paper presents an efficient vector system for plant transformation experiments. A first component is the avirulent acceptor Ti plasmid pGV2260 in which the entire T-DNA region is substituted by pBR322 sequences. The second component is pGV831, a Sm^R/Sp^R pBR derivative containing the border sequence of the octopine TL-DNA, the neomycin phosphotransferase gene under control of the nopaline synthase promoter, and a BamHI site for cloning DNA of interest in this T-DNA.

This vector system overcomes the tedious work of isolating double recombinants between vector and Ti plasmid. A cointegrate Ti plasmid is formed upon simple mobilization of pGV831-derived plasmids into Agrobacterium containing pGV2260. The Sp^R/Sm^R marker allows efficient selection of the recombinants. The cointegrates contain the pGV831 T-DNA flanked by directly repeated pBR sequences. Cointegrates formed with pGV3850 have direct-repeats of pBR322 within the T-region [7]. The insertion of the repeated pBR322 copies in the plant DNA increases the size of T-DNA by at least 10 kb and often complicates its physical analysis.

An approach to avoid recombinational steps is the use of binary vectors in which T-DNA is carried on one replicon and its transfer to the plant cell is complemented by Ti plasmid vir genes located on a second plasmid [33-35]. However, a systematic analysis to investigate whether the copy number and the precision of T-DNA integration are identical to the wild-type situation has not been performed yet. The instability of presently available binary vectors is still an important consideration in their use [35].

We have used our vector system to transform tobacco protoplasts, using the Km^R gene as selectable marker. Km^R calli were obtained reproducibly at high frequencies, and transformed plants could easily be regenerated from such clones. Selection for kanamycin resistance greatly facilitates the identification of transformants if compared to the screening for nopaline-positive clones as was done for pGV3850-transformed cells [7]. Furthermore, selection will be the only practical procedure when transforming cells derived from plants that can only be transformed at a much lower frequency as compared to tobacco cells.

The DNA of transformed calli and of regenerated plants has been analyzed by Southern hybridization. The results indicate that (i) the T-DNA border sequences present in the vector are used as integration signals since T-DNA/plant DNA junctions occur at the border fragments; (ii) the

number of integrated T-DNA copies per genome is low (3 to 4) and is similar to that in wild-type pTiB6S3 tumor lines [36]. In recent experiments performed in our laboratory, pGV831-derived vectors have been used to transfer different genes into plants. The extent and the copy number of T-DNA have been determined in several tens of them. The vast majority was also delineated by the border sequences and present in 1 to 5 copies [M. De Beuckeleer, unpublished observations]. This generalizes the observations we have made in this work.

In a considerable fraction of cell lines transformed with pGV2435 no octopine synthase activity could be detected. Analysis of two such Ocs$^-$ lines did not reveal any aberrancies in the integrated T-DNA, as a matter of fact the internal T-DNA fragment containing the ocs gene was present in the plant genome. Also, transformed plants regenerated from the two Ocs$^-$ lines did not produce octopine. This illustrates the variability in the level of gene expression when genes are introduced into plant cells. The factors influencing expression are unclear. Position effect due to insertions in different sites of the genome are not likely to be the only reason for the variability since little or no correlation exists between expression of ocs and the resistance to kanamycin, although both genes are separated only by 600 bp.

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REFERENCES

1. Nester, E.W., Gordon, M.P., Amasino, R.M. and Yanofsky, M.F. (1984) Annu. Rev. Plant Physiol. 35, 387-413.
2. Gheysen, G., Dhaese, P., Van Montagu, M. and Schell, J. (1985) in Genetic flux in plants, Hohn, B. and Dennis, E.S. Eds., (Advances in Plant Gene Research, Vol. 2), in press. Wien, Springer-Verlag.
3. Caplan, A., Herrera-Estrella, L., Inzé, D., Van Haute, E., Van Montagu, M., Schell, J. and Zambryski, P. (1983) Science 222, 815-821.
4. Hooykaas-Van Slogteren, G.M.S., Hooykaas, P.J.J. and Schilperoort, R.A. (1984) Nature (London) 311, 763-764.
5. Hernalsteens, J.-P., Thia-Toong, L., Schell, J. and Van Montagu, M. (1984) EMBO J. 3, 3039-3041.
6. Leemans, J., Deblaere, R., Willmitzer, L., De Greve, H., Hernalsteens, J.P., Van Montagu, M. and Schell, J. (1982) EMBO J. 1, 147-152.
7. Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M. and Schell, J. (1983) EMBO J. 2, 2143-2150.

8. Hille, J., Wullems, G. and Schilperoort, R. (1983) Plant Mol. Biol. 2, 155-163.
9. Wang, K., Herrera-Estrella, L., Van Montagu, M. and Zambryski, P. (1984) Cell 38, 455-462.
10. De Block, M., Herrera-Estrella, L., Van Montagu, M., Schell, J. and Zambryski, P. (1984) EMBO J. 3, 1681-1689.
11. Horsch, R.B., Fraley, R.T., Rogers, S.G., Sanders, P.R., Lloyd, A. and Hoffman, N. (1984) Science 223, 496-498.
12. Zambryski, P., Herrera-Estrella, L., De Block, M., Van Montagu, M. and Schell, J. (1984) in Genetic engineering, Principles and Methods, Setlow, J. and Hollaender, A. Eds., Vol. 6, pp. 253-278. Plenum Press, New York.
13. Herrera-Estrella, L., Depicker, A., Van Montagu, M. and Schell, J. (1983) Nature (London) 303, 209-213.
14. Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J.-P., Van Montagu, M. and Schell, J. (1983) EMBO J. 2, 987-995.
15. Bevan, M.W., Flavell, R.B. and Chilton, M.-D. (1983) Nature (London) 304, 184-187.
16. Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C. (1983) Proc. Natl. Acad. Sci. USA 80, 4803-4807.
17. Colson, C., Glover, S.W., Symonds, N. and Stacey, K.A. (1965) Genetics 52, 1043-1050.
18. Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I., Schilperoort, R.A. and Schell, J. (1974) Nature (London) 252, 169-170.
19. Bolivar, F., Rodriguez, R., Greene, P., Betlach, M., Heynecker, H., Boyer, H., Crosa, J. and Falkow, S. (1977) Gene 2, 95-113.
20. Prentki, P., Karch, F., Iida, S. and Meyer, J. (1981) Gene 14, 289-299.
21. Hohn, B. and Collins, J. (1980) Gene 11, 291-298.
22. Rao, R.N. and Rogers, S.G. (1979) Gene 7, 79-82.
23. Leemans, J., Shaw, C., Deblaere, R., De Greve, H., Hernalsteens, J.P., Maes, M., Van Montagu, M. and Schell, J. (1981) J. Mol. Appl. Genet. 1, 149-164.
24. De Greve, H., Decraemer, H., Seurinck, J., Van Montagu, M. and Schell, J. (1981) Plasmid 6, 235-248.
25. Van Haute, E., Joos, H., Maes, M., Warren, G., Van Montagu, M. and Schell, J. (1983) EMBO J. 2, 411-418.
26. O'Farrell, P.H., Kutter, E. and Nakanishi, M. (1980) Mol. Gen. Genet. 179, 421-435.
27. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York.
28. Márton, L., Wullems, G.J., Molendijk, L. and Schilperoort, R.A. (1979) Nature (London) 277, 129-130.
29. Murashige, T. and Skoog, F. (1962) Physiol. Plantarum 15, 473-497.
30. Thomashow, M.F., Nutter, R., Montoya, A.L., Gordon, M.P. and Nester, E.W. (1980) Cell 19, 729-739.
31. Leemans, J., Langenakens, J., De Greve, H., Deblaere, R., Van Montagu, M. and Schell, J. (1982) Gene 19, 361-364.
32. Vieira, J. and Messing, J. (1982) Gene 19, 259-268.
33. Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) Nature (London) 303, 179-181.
34. de Framond, A.J., Barton, K.A. and Chilton, M.-D. (1983) Bio/technology 1, 262-269.
35. Bevan, M.W. (1984) Nucl. Acids Res. 12, 8711-8721.
36. De Beuckeleer, M., Lemmers, M., De Vos, G., Willmitzer, L., Van Montagu, M. and Schell, J. (1981) Mol. Gen. Genet. 183, 283-288.